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Anti-pneumococcal surface protein A antibodies transferred from maternal mice protect offspring against lethal infection with *Streptococcus pneumoniae* serotype 3

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Streptococcus pneumoniae (*S. pneumoniae*) causes otitis media, pneumonia, and invasive pneumococcal diseases (IPDs) such as meningitis and septicemia in humans. IPDs are fatal in children and can cause irreversible sequelae such as brain damage and impaired hearing. The introduction of 7- and 13-valent pneumococcal, conjugate, polysaccharide-based vaccines (PCV7, Prevnar 7®; and PCV13, Prevnar 13®) has decreased pediatric IPD infections. However, PCV13 provides limited protection against *S. pneumoniae* serotype 3. A new candidate pneumococcal vaccine antigen, pneumococcal surface protein A (PspA), which is found in almost all *S. pneumoniae* serotypes, addresses the drawbacks of CPS-based vaccines. In a previous study, the PspA3 + 2 protein was developed as a broad-spectrum vaccine candidate that combines PspA clades 2 and 3. We assessed whether vaccinating pregnant mice with PspA3 + 2 would transfer anti-PspA3 + 2 antibodies to pups, and if so, whether the transferred antibodies would protect against bacteremia caused by *S. pneumoniae* serotype 3. A PspA3 + 2 vaccine containing aluminum hydroxide gel (alum) and cytosine-phosphate-guanosine oligodeoxynucleotide K3 adjuvants induced anti-PspA antibodies in adult female mice, and an enzyme-linked immunosorbent assay revealed anti-PspA antibodies in serum samples from their offspring. Survival rates after lethal infection with *S. pneumoniae* serotype 3 were significantly higher among these neonates than in negative controls. These findings suggest that anti-PspA3 + 2 antibodies transferred from maternal mice vaccinated with PspA3 + 2 protect against bacteremia caused by *S. pneumoniae* serotype 3 in newborn pups.

KEYWORDS

***Streptococcus pneumoniae*, vaccine, lethal bacteremia, prenatal antibody transfer, PspA**

1 Introduction

Streptococcus pneumoniae (*S. pneumoniae*) causes otitis media, pneumonia, and invasive pneumococcal diseases (IPDs) such as meningitis and septicemia in humans. Pneumococcal meningitis in children is a threatening infectious disease that can be fatal and has sequelae such as brain damage and impaired hearing (Baraff et al., 1993; Jit, 2010; Shinjoh et al., 2014; Wahl et al., 2018; El Kareh et al., 2020).

The 7- and 13-valent pneumococcal conjugate vaccines (PCV7, Prevnar7®; and PCV13, Prevnar 13®), which use capsular polysaccharide (CPS) as a vaccine antigen, were introduced into Japan's routine vaccination program for children in 2010 and 2013, respectively. A comparison of the number of pediatric pneumococcal meningitis cases in Japan before (2008-2012) and after (2014-2016) the introduction of routine PCV7 vaccination revealed a 70.3% decrease (Iwata et al., 2021). However, in recent years, among 34 reported cases of pediatric pneumococcal meningitis post-PCV13 introduction, the majority (30/34) were caused by non-PCV13 serotypes. Notably, all cases with sequelae (6 cases) or fatal outcomes (4 cases) were attributable to non-PCV13 serotypes (Kurihara et al., 2022). Furthermore, 9 of the 34 *S. pneumoniae* isolates that caused pediatric pneumococcal meningitis were penicillin-resistant and belonged to non-PCV13 serotypes (Kurihara et al., 2022). These findings highlight the increasing incidence of infections caused by non-PCV13 pneumococcal serotypes and the emergence of antibiotic-resistant *S. pneumoniae* as critical clinical concerns (Iwata et al., 2021; Kurihara et al., 2022).

Thus, new CPS-based vaccines have been developed, such as the 15-valent pneumococcal conjugate vaccine (PCV15, VAXNEUVANCE®) and 20-valent pneumococcal conjugate vaccine (PCV20, Prevnar 20®) (Peterson et al., 2019; Hurley et al., 2021). The trend of pediatric IPDs caused by serotypes not covered by PCV13 has continued. Therefore, routine pneumococcal vaccination with PCV13 transitioned to PCV15 and PCV20 in Japan during 2024 (Suga et al., 2015; Kurihara et al., 2022). However, CPS is the target of the vaccine, which is associated with the risk that serotype substitution could result in the emergence of non-target serotypes. In addition to serotype replacement, the effectiveness of PCV13 against *S. pneumoniae* serotype 3 might be limited (Groves et al., 2019; Domínguez et al., 2017; Azarian et al., 2018; Deceuninck et al., 2023; Calvo-Silveria et al., 2024; Silva-Costa et al., 2022). *S. pneumoniae* serotype 3 has thicker capsules than do other serotypes (except serotype 37), and despite being covered by PCV13, it remains a major cause of pneumococcal infectious diseases (Luck et al., 2020).

The proportion of diseases caused by *S. pneumoniae* serotype 3 has notably not decreased, despite serotype 3 CPS being included in PCV13 (Azarian et al., 2018; Katoh et al., 2017; Silva-Costa et al., 2022; Wijayasri et al., 2019; Lansbury et al., 2024; Calvo-Silveria et al., 2024). Pneumococcal pneumonia with pleural effusion or pulmonary edema caused by *S. pneumoniae* serotype 3 remains a significant concern in Germany, where routine PCV13 vaccination for children has been implemented (Goettler et al., 2020). *S. pneumoniae* serotype 3 has caused more septic shock than have other serotypes in Sweden (Ahl et al., 2013). Major cardiovascular adverse events are more frequent among Colombian patients with IPDs caused by *S. pneumoniae* serotype 3 (Africano et al., 2021). The limited effects of PCV13 against *S. pneumoniae* serotype 3 have been attributed to serotype 3 CPS not being covalently attached to the bacteria surface, which allows it to shed (Cartee et al., 2005; Choi et al., 2016). Consequently, more anti-CPS antibodies are required to protect against IPDs caused by *S. pneumoniae* serotype 3 than by other *S. pneumoniae* serotypes (Choi et al., 2016; Andrews et al., 2014).

Pneumococcal surface protein A (PspA) is a candidate antigen for a CPS-independent pneumococcal vaccine that can overcome the deficiencies of CPS-based vaccines (McDaniel et al., 1991; Tart et al., 1996; Xin et al., 2009; Piao et al., 2014; Fukuyama et al., 2015). PspA functions in evading host immune defenses by preventing killing via host-secreted lactoferrin, escaping destruction via neutrophil extracellular traps, inhibiting C3 complement deposition, and facilitating adhesion to glyceraldehyde-3-phosphate dehydrogenase (Hammerschmidt et al., 1999; Tu et al., 1999; Ren et al., 2003; Daniels et al., 2006; Hollingshead et al., 2006; Martinez et al., 2019; Park et al., 2021). PspA comprises the following five domains: a signal peptide, an α-helical highly charged domain (α-HD), a proline-rich domain (PRD), a choline-binding domain, and a short hydrophobic tail. The C-terminal 100 amino acids of the α-HD form a clade-defining region (CDR). PspA is classified into three families and six clades based on the amino acid sequence of its CDR (family 1, clades 1 and 2; family 2, clades 3–5; and family 3, clade 6) (Hollingshead et al., 2000). The PRD of PspA is classified into three groups, PRD1–3, according to its amino acid sequence (Mukerji et al., 2018). Approximately 98% of pneumococcal strains from adult patients with IPDs in Japan express PspA clades 1–4 (Chang et al., 2021). The PspA3 + 2 vaccine antigen was developed considering cross-reactivity, as a single PspA does not provide sufficient protection against lethal infection by *S. pneumoniae* of another PspA clade family (Darrieux et al., 2007; Piao et al., 2014; Genschmer et al., 2019). This vaccine combines the α-HDs and PRDs from TIGR4 (PspA clade 3/PDR 1) and WU2 (PspA clade 2/PDR 3) and has been shown to protect mice from lethal infection by *S. pneumoniae* expressing PspA clades 1–5 (Piao et al., 2014).

Preventing IPDs in children with undeveloped immune systems is challenging. We therefore hypothesized that vaccinating prenatal mice with PspA3 + 2 would generate anti-PspA3 + 2 immunoglobulin G (IgG) antibodies that could be transferred to neonates, thus providing protection against infection. Maternal antibodies induced by PspA-based vaccines have protected infant mice against pneumococcal infection (Katsurahara et al., 2008; Hotomi et al., 2011; Kono et al., 2011, Kono et al., 2023). In these studies, cholera toxin was used as an adjuvant for nasal immunization. While cholera toxin is a potent mucosal adjuvant, adverse effects have been reported (Lewis et al., 2009). In this study, unlike previous studies, we evaluated the protective efficacy of a PspA-based vaccine using an alternative adjuvant suitable for intramuscular (i.m.) administration. Specifically, we investigated

the protective effect of a PspA3 + 2 vaccine formulated with aluminum salts, which are used in PCV13 and other vaccines, and cytosine-phosphate-guanine oligodeoxynucleotide (CpG-ODN) K3, a toll-like receptor ligand with confirmed tolerability (Verthelyi et al., 2001; Pfizer Inc., 2010; Ezoe et al., 2020; Otsuka et al., 2022) and ability to induce anti-PspA antibodies as an adjuvant (Piao et al., 2014; Yokota et al., 2023). The present study provides insights into the clinical applications of the PspA-based vaccine, including the use of CpG-ODN K3 adjuvants.

2 Material and methods

2.1 Bacterial strains

Streptococcus pneumoniae WU2 (serotype 3/PspA clade 2/PRD 3) was incubated at 37°C under a 5% CO_2 atmosphere in trypticase soy agar with 5% sheep blood (TSA II; BD, Franklin Lakes, NJ, USA) (Hollingshead et al., 2000; Piao et al., 2014) and in Todd-Hewitt broth supplemented with 0.5% yeast extract (THYB; BD).

2.2 Recombinant PspA protein purification

The construction of the pET28a (+) vector encoding the PspA3 + 2 protein and the purification of recombinant PspA3 + 2 were performed as described previously (Piao et al., 2014; Yokota et al., 2023). Briefly, *Escherichia coli* BL21 was transformed with the pET28a (+) vector encoding PspA3 + 2 and cultured in LB medium (Merck, Darmstadt, Germany) supplemented with 50 μg/mL kanamycin and Overnight Express™ Autoinduction System 1 (Merck) according to the manufacturer's instructions. The bacterial culture was centrifuged, and the cell pellet was washed with phosphate buffered saline (PBS) before being resuspended in PBS (pH 8.0) containing 10 mM imidazole (Nacalai Tesque, Inc., Kyoto, Japan) and a protease inhibitor cocktail (Merck). The cells were lysed via sonication, and the lysate was centrifuged to collect the supernatant, which was subsequently filtered through a 0.45-μm membrane filter (Merck). The filtrate was purified using a His-Trap HP column (Cytiva, Marlborough, MA, USA) installed on an NGC Quest 10 Chromatography System (Bio-Rad, Hercules, CA, USA). The PspA3 + 2 protein was collected, concentrated using an Amicon Ultra-15 10 K device (Merck), and further purified with a HiLoad 26/600 Superdex 200 pg column (Cytiva). PspA3 + 2 concentration was determined using a bicinchoninic acid protein assay kit (Takara Bio, Inc., Kusatsu, Japan). PspA3 + 2 was used for mouse immunization and enzyme-linked immunosorbent assay (ELISA).

2.3 ELISA for anti-PspA antibody

We performed ELISA based on previously described protocols (Morino et al., 2020; Kuroda et al., 2023). Ninety-six-well plates (Greiner Bio-One, Frickenhausen, Germany) with a carbonate–bicarbonate buffer (Fujifilm Wako Pure Chemical Corp., Osaka, Japan) were coated with 1 μg/mL recombinant PspA3 + 2. Mouse serum samples (mother mouse serum 1:400 initial dilution, pooled infant mouse serum 1: 200 initial dilution) and goat anti-mouse IgG horseradish peroxidase secondary antibody (1:10,000; Abcam, Cambridge, UK) were used in this assay. The optical density (OD) was measured at 450 nm using an ELISA plate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA). Reciprocal $\log_2$ titers were determined based on the highest antibody dilution that registered an OD of 0.1.

2.4 Animal studies

Nine-to-twelve-week-old male adult BALB/c and five-week-old female adult BALB/c were obtained from Japan SLC, Inc. (Shizuoka, Japan). Male mice were only used for mating. After one week of housing, the female mice were immunized via i.m. injections of 5 μg alum (Fujifilm Wako Pure Chemical Corp.) and 2.5 μg CpG-ODN K3 (Ajinomoto Bio-Pharma Services Platform Technologies, Osaka, Japan) with or without (negative controls) recombinant PspA3 + 2 protein (0.1 μg) into their thigh muscles three times at 7-day intervals. The vaccinated and negative control female mice were mated, and newborn pups were housed under the same rearing conditions. Serum samples were collected from the postnatal mice (but not immediately before or after delivery), and from the pups on days 0 (birth day), 4, 9, and 14 starting from 1 week after the last vaccination to determine anti-PspA antibody titers. Serum samples from littermates were pooled according to their mother and date of birth (Table 1).We intraperitoneally (i.p.) injected 8-day-old (infant) mice born to seven immunized mice and five negative control mice with five colony-forming units of *S. pneumoniae* serotype 3. We then assessed survival rates 6 days later to determine the protective performance of the vaccine.

The Institutional Animal Care and Use Committee at the Graduate School of Medicine, Osaka University, Japan approved all the animal experiments (Approval ID: 04-021-005).

TABLE 1 Pooling of serum samples from littermates.

Group	ID	Number of littermates			
		Day 0	Day 4	Day 9	Day 14
Prenaral vaccinated	No. 1	2	2	1	2
	No. 2	3	2	1	1
	No. 3	2	2	1	1
	No. 4	0 (sampling error)	1	1	1
	No. 5	2	1	1	1
Negative control	No. 1	1	1	1	1
	No. 2	3	3	2	1
	No. 3	3	2	2	1

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2.5 Statistical analysis

Quantitative data are presented as means and standard errors. Data were statistically analyzed using GraphPad Prism 8 (GraphPad Software Inc., Boston, MA, USA).

3 Results

3.1 Antibody titer of the PspA3 + 2 vaccine in immunized maternal mice

Maternal mice received three i.m. injections at 1-week intervals with alum and CpG-ODN K3 without (negative controls) or with (vaccinated) PspA3 + 2 (Figure 1A). Serum samples were obtained from the vaccinated and negative control mice at intervals of 21–100 days after the first immunization (Figure 1A). IgG antibody titers against the PspA3 + 2 protein in the sera of vaccinated and negative control mice were measured using ELISA and then compared using software (Figure 1B). The results revealed higher titers in the vaccinated mice than in the control mice.

3.2 Antibody titers of offspring born to PspA3 + 2-vaccinated mice

We assessed anti-PspA antibody transfer from vaccinated mice to 0-, 4-, 9-, and 14-day-old offspring (Figure 2A). IgG antibody titers against the PspA3 + 2 protein in serum samples were analyzed using ELISA (Figure 2B). The results revealed significantly increased antibody titers in infants delivered by vaccinated mice compared with those in negative controls, excluding 0-day-old offspring (Figure 2B).

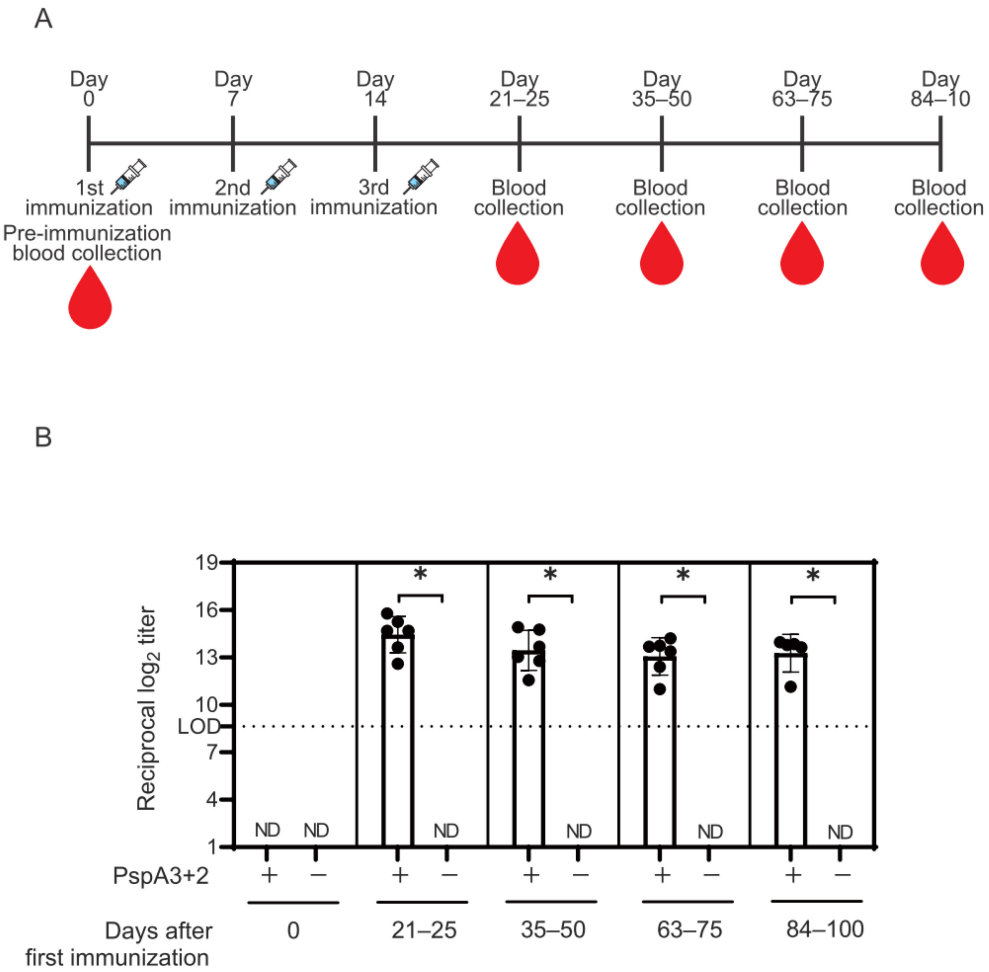

FIGURE 1
Titers of IgG antibodies against the PspA3 + 2 protein in the sera of prenatal mice. **(A)** Immunization schedule and blood collected from prenatal mice. Day 0: time point immediately before first immunization. **(B)** Titers of IgG antibodies against PspA3 + 2 in the sera of prenatal vaccinated (n = 6) and negative controls (n = 3) determined using ELISA. One prenatal mouse was missing between days 84–100 due to sampling error. The initial dilution of mouse serum used in the ELISA was 1:400. The limit of detection (LOD) was determined as $\log_2(400) = 8.64$ for 1:400. Error bars indicate standard error of the mean. ND, not detected. The IgG antibody titer of the mouse serum was measured in a single independent experiment. Statistically significant: $^*p < 0.05$ (Mann-Whitney *U*-test). In the Mann-Whitney *U*-test, the negative controls were below the LOD; therefore, statistical analysis was performed using a twofold dilution of the initial serum dilution, $\log_2(400/2)=7.64$.

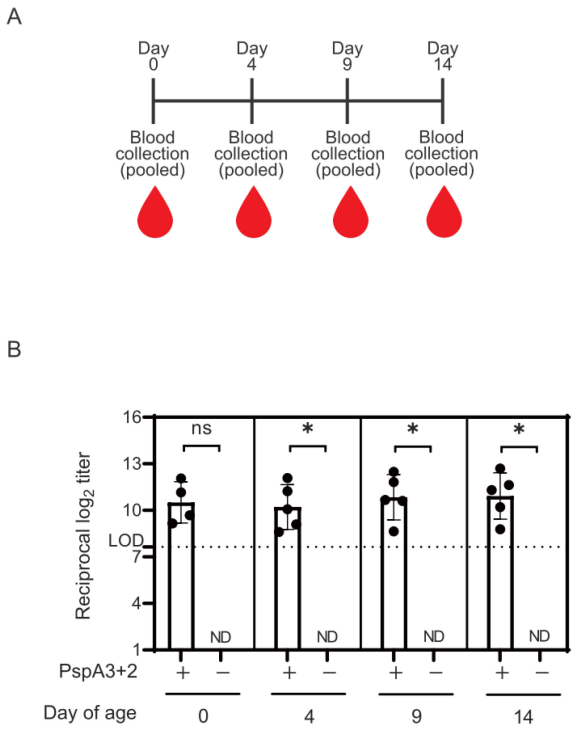

FIGURE 2
Titers of IgG antibodies against the PspA3 + 2 protein in the sera of infant mice. **(A)** Schedule of blood collection from infant mice. **(B)** Date of birth was taken as day 0. Titers of IgG antibodies against PspA3 + 2 in the sera of neonatal mice at birth and 4, 9, and 14 days thereafter determined using ELISA. Serum samples from littermates were pooled according to their mother and date of birth. The initial dilution of mouse serum used in the ELISA was 1:200. The limit of detection (LOD) was determined as $\log_2(200) = 7.64$ for an initial serum dilution of 1:200. Error bars indicate standard errors of means. ND, not detected. The IgG antibody titer of the mouse serum was measured in a single independent experiment. Statistically significant: $*p < 0.05$, non-significant (ns): $p \geq 0.05$ (Mann-Whitney *U*-test). In the Mann-Whitney *U*-test, the negative controls were below the LOD; therefore, statistical analysis was performed using a twofold dilution of the initial serum dilution, $\log_2(200/2)=6.64$.

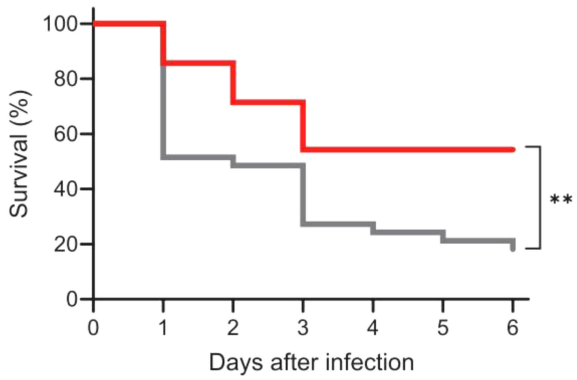

FIGURE 3
Protection against lethal infection by *Streptococcus pneumoniae* WU2 serotype 3 in mice with maternal anti-PspA3 + 2 antibodies. Eight-day-old mice were intraperitoneally injected with *S. pneumoniae* serotype 3 WU2, and the survival rates were monitored for 6 days. Red and gray lines indicate infant mice delivered by pregnant mice vaccinated with (n = 35) or without (negative controls; n = 33) PspA3 + 2, respectively. Comparison of survival rates of lethal *S. pneumoniae* serotype 3 WU2 infections between immunized and negative control mice. Statistically significant $**p < 0.01$ (log-rank test).

3.3 Ability of transferred IgG antibodies to protect infant mice against lethal *S. pneumoniae* serotype 3 infection

We investigated whether anti-PspA antibodies could protect infant mice against bacteremia caused by *S. pneumoniae* serotype 3 WU2 expressing PspA clade 2. We compared survival rates between infants born to vaccinated and unvaccinated (negative controls) mice administered lethal doses of *S. pneumoniae* WU2 i.p. Survival rates were significantly higher among infants born to the vaccinated mice than among the negative controls (54.3% vs. 18.2%; Figure 3).

4 Discussion

We vaccinated pregnant mice with a recombinant PspA3 + 2 protein with alum and CpG-ODN K3 as adjuvants. Maternal anti-PspA3 + 2 antibodies were transferred to offspring, providing partial protection against fatal pneumococcal bacteremia caused by *S. pneumoniae* serotype 3.

In this study, the PspA3 + 2 vaccine consisted of 0.1 μg of PspA3 + 2, 2.5 μg of CpG-ODN K3, and 5 μg of alum administered via i.m. injection. This dosage and route of administration were selected based on previous studies in adult mice, in which they induced a robust antibody response and provided protection against lethal *S. pneumoniae* infection (Piao et al., 2014). Following a similar approach, we investigated the protective effect of maternal i.m. immunization with the PspA3 + 2 vaccine on offspring against *S. pneumoniae* infection. This study also aimed to evaluate an immunization strategy with potential applicability to humans. CpG-ODN K3 was chosen as an adjuvant in part due to its demonstrated tolerability in humans (Ezoe et al., 2020; Otsuka et al., 2022).

Previous studies on PspA-based vaccines have utilized antigen doses ranging from 1–20 μg and employed subcutaneous (s.c.) or intranasal (i.n.) administration (Vadesilho et al., 2014; Kuipers et al., 2015; Tamborrini et al., 2015; Converso et al., 2017; Goulart et al., 2017; Kono et al., 2023; Yokota et al., 2023). In this study, the antigen dose (0.1 μg) was considerably lower; therefore, increasing the antigen and adjuvant doses may enhance maternal antibody production, improving protection in offspring. S.c. and i.n. administration routes may also have potential for inducing immunity. Future studies comparing different doses, adjuvants, and administration routes will be necessary to further optimize the immunization strategy.

A potential limitation of this study is that the level of lipopolysaccharide (LPS) contamination in the recombinant protein preparations was not assessed. Although our purification steps, including affinity and size-exclusion chromatography, likely reduced LPS contamination, we cannot exclude the possibility that

residual LPS influenced the experimental outcomes. In future studies, LPS removal strategies and quantitative assays, such as the Limulus amebocyte lysate assay, will be implemented to ensure that LPS contamination does not confound the results.

S. pneumoniae colonizes the nasopharynx of infants and young children, with rates of approximately 50% in Japan (Hashida et al., 2011; Chang et al., 2020; Otsuka et al., 2013) and 90% in Finland (Holmlund et al., 2006). Children naturally become sensitized to *S. pneumoniae* through routine PCV13 vaccination and nasopharyngeal carriage and gradually acquire antibodies against pneumococcus as they age. For example, the level of anti-PspA IgG is the lowest in infants aged 6–11 months and highest in preschool and school-aged children (Morino et al., 2020). However, children lack protective immunity during a vulnerable period before receiving PCV13 or natural sensitization. Since approximately 60% of mothers in developed countries breastfeed their offspring for 6 months, anti-PspA antibodies in breast milk and transferred via the placenta can protect against *S. pneumoniae* during this period (Hotomi et al., 2011; Victora et al., 2016).

In Japan, an investigation of 39 pediatric pneumococcal meningitis cases from 2014 to 2016 revealed that 34 were caused by non-PCV13 *S. pneumoniae* serotypes (34/39) (Iwata et al., 2021). Among these, 13 were attributable to PCV20-covered serotypes, 13 to non-PCV20 serotypes, and 8 to serotype 15 (15A, 15B, or 15C) (Iwata et al., 2021). These findings suggest that even with the introduction of PCV20, non-PCV20 serotypes of *S. pneumoniae* remain a threat. PCV20 is expected to be somewhat effective against serotype replacement; however, future vaccines should either include a broader range of serotypes or target antigens common to all *S. pneumoniae* strains. The development of PspA-based candidates for a universal pneumococcal vaccine is promising for controlling pneumococcal infections. Predicting the occurrence of bacteremia in children is challenging, but if maternally transferred antibodies can prevent severe pneumococcal bacteremia, then administering a PspA-based vaccine to prenatal women might prevent bacteremia in children.

Data availability statement

The datasets presented in this article are not readily available because they were obtained from third-party sources and are subject to ownership and distribution rights held by the original providers. The sources of these materials are cited in the manuscript. Requests to access the datasets should be directed to the corresponding author, and access will be facilitated by coordinating with the original providers as appropriate.

Ethics statement

The animal study was approved by The Institutional Animal Care and Use Committee at the Graduate School of Medicine, Osaka University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

EK: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. YT: Data curation, Methodology, Writing – review & editing. SH: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

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